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Production and Targeting of the *Brucella abortus* Antigen L7/L12 in *Lactococcus lactis*: a First Step towards Food-Grade Live Vaccines against Brucellosis

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ABSTRACT

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Brucella abortus is a facultative intracellular gram-negative bacterial pathogen that infects humans and animals by entry mainly through the digestive tract. *B. abortus* causes abortion in pregnant cattle and undulant fever in humans. The immunogenic *B. abortus* ribosomal protein L7/L12 is a promising candidate antigen for the development of oral live vaccines against brucellosis, using food-grade lactic acid bacteria (LAB) as a carrier. The *L7/L12* gene was expressed in *Lactococcus lactis*, the model LAB, under the nisin-inducible promoter. Using different signals, L7/L12 was produced in cytoplasmic, cell-wall-anchored, and secreted forms. Cytoplasmic production of L7/L12 gave a low yield, estimated at 0.5 mg/liter. Interestingly, a secretable form of this normally cytoplasmic protein via fusion with a signal peptide resulted in increased yield of L7/L12 to 3 mg/liter; secretion efficiency (SE) was 35%. A fusion between the mature moiety of the staphylococcal nuclease (Nuc) and L7/L12 further increased yield to 8 mg/liter. Fusion with a synthetic propeptide (LEISSTCDA) previously described as an enhancer for heterologous protein secretion in *L. lactis* (Y. Le Loir, A. Gruss, S. D. Ehrlich, and P. Langella, J. Bacteriol. 180:1895-1903, 1998) raised the yield to 8 mg/liter and SE to 50%. A surface-anchored L7/L12 form in *L. lactis* was obtained by fusing the cell

wall anchor of *Streptococcus pyogenes* M6 protein to the C-terminal end of L7/L12. The fusions described allow the production and targeting of L7/L12 in three different locations in *L. lactis*. This is the first example of a *B. abortus* antigen produced in a food-grade bacterium and opens new perspectives for alternative vaccine strategies against brucellosis.

INTRODUCTION

Brucellosis remains a worldwide zoonosis that causes abortion and infertility in cattle. It also causes undulant fever, endocarditis, arthritis and osteomyelitis in humans (for a review, see reference 3). Infection in humans occurs through direct contact with infected animals and from ingestion of contaminated dairy products. *Brucella abortus*, a facultative intracellular gram-negative bacterium, is the causative agent (49). Vaccination against brucellosis in cattle and in wild ungulates relies on live attenuated strains such as *B. abortus* strain 19 (6, 7) or strain RB51 (6). Although efficient in promoting a protective immune response, strain 19 is pathogenic for humans and provokes abortion when administered to pregnant cattle (6). Although strain RB51 is considered the vaccine of choice against brucellosis for cattle in the United States, it is derived from a virulent *B. abortus* 2308 strain and is not, like strain 19, recommended for pregnant animals (6, 18, 43). Furthermore, there are no human vaccines currently available, and the use of a food-grade oral vaccine expressing a common antigen from *B. abortus* and *Brucella melitensis* would be an alternative approach to immunize humans and animals against brucellosis.

Alternative vaccination strategies using *B. abortus* antigens with characterized immunogenic properties have recently been investigated (1, 29, 36, 44). Live vaccine strategies are being optimized using isolated antigens produced in either recombinant attenuated *B. abortus* strains (44, 45) or in a live *Escherichia coli* strain (16, 31). An effective vaccine against intracellular pathogens requires a cell-mediated immune response. An antigen of *B. abortus* that elicits a cell-mediated immune response and confers protective immunity in mice is the ribosomal protein L7/L12 (30).

The use of live, food-grade, noninvasive, nonpathogenic lactic acid bacteria (LAB) as antigen delivery vehicles is a promising vaccine strategy. This strategy could overcome potential problems due to the use of live *B. abortus* strains as antigen delivery vehicles (such as cross-reaction with diagnostic test, residual virulence, and reversion risks), and it provides a means for large scale and low-cost vaccine administration. To date, several bacterial and viral antigens have already been produced in *Lactococcus lactis*, the model LAB (15, 17, 21, 34, 48); in some cases, immunogenicity was demonstrated (12, 27, 35). These encouraging results suggest the feasibility of the LAB-based vaccine approach. Note that a protective immune response depends not only on the antigen and the delivery vehicle, but also on the location of the antigen (25). In some cases, antigen export may be of interest since it allows a direct contact between the antigen and the immune system.

In this work, we constructed *L. lactis* strains that produce recombinant *B. abortus* L7/L12 antigen. High L7/L12 levels were obtained using an inducible promoter (9). Three different expression vectors were

used to target the protein to cytoplasm, surface, or culture medium. In the case of exported L7/L12, both secretion efficiency and yield were improved by its fusion to the staphylococcal nuclease (Nuc), a well-secreted reporter enzyme (38), or to a synthetic propeptide (23).

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Bacterial strains, growth conditions, and DNA manipulations. *L. lactis* NZ9000 (19) was grown in M17 medium (Difco [41]) supplemented with 1% glucose or in brain heart infusion (Difco) at 30°C without agitation. *E. coli* TG1 (14) was grown in aerated Luria-Bertani (37) medium at 37°C. Unless otherwise indicated, plasmid constructions were first established in *E. coli* and then transferred to *L. lactis*. Electrotransformation was performed as previously described (21). Plasmids were selected by addition of antibiotics as follows (concentrations in micrograms per milliliter): for *L. lactis*, erythromycin (5) or chloramphenicol (10); and for *E. coli*, ampicillin (100) or chloramphenicol (10). When both erythromycin and ampicillin were used for *E. coli*, concentrations were 75 μg/ml for each antibiotic. DNA manipulations were performed as described (37).

Cloning of the *L7/L12* gene under control of nisin-inducible promoter P_{nisA}. The different *L7/L12* expression cassettes are described in Fig. 1 and plasmids used in this work are listed in Table 1. Constructions were checked by DNA sequencing. Dideoxynucleotide chain termination DNA sequencing reactions were carried out on double-stranded DNA with the Taq Dye Primer Cycle Sequencing kit (Applied Biosystems, San Jose, Calif.) using a Perkin-Elmer PCR apparatus. Sequencing reactions were primed with fluorescent oligonucleotides and analyzed on a model 370A DNA sequencer (Applied Biosystems).

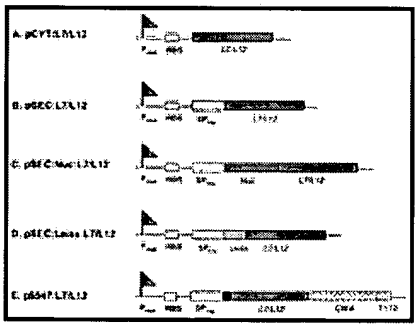

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FIG. 1. Expression cassettes for L7/L12 production and export using the nisin-inducible promoter (P_{nisA}) and the lactococcal signal peptide SP_{Usp}. Schematic structures of the fusion proteins carried by the indicated plasmids. For details of plasmid construction, see Materials and Methods and Table 1. P_{nisA}, nisin-inducible promoter; RBS, ribosomal binding site of the *usp45* gene; SP_{Usp}, signal peptide of Usp45; Nuc, staphylococcal nuclease; Leiss, sequence encoding the LEISSTCDA synthetic propeptide; black bar, 11 first amino acids of Usp45; L7/L12, DNA fragment encoding the mature L7/L12 protein; CWA, sequence encoding the cell wall M6 protein; T1T2, termination signal of *L. lactis* tryptophan gene (no to scale).

View this table: TABLE 1. Plasmids used in this study
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Plasmids pCYT:Nuc, pSEC:Nuc, pSEC:LEISS:Nuc, and pVE5547 (Table 1) were used to clone the *L7/L12* gene under the transcriptional control of lactococcal nisin-inducible promoter P_{nisA} (9). pSEC:Nuc and pCYT:Nuc are derivatives of pNZ8010 in which the *gus* gene is under the transcriptional control of P_{nisA}. To construct pSEC:Nuc, the *gus* gene was replaced by a *Bam*HI-*Xho*I-cut DNA fragment encoding the ribosomal binding site (RBS_{Usp}) and signal peptide (SP_{Usp}) of the *usp45* gene and the mature part of Nuc. To construct pCYT:Nuc, the DNA fragment encoding SP_{Usp} was deleted by reverse PCR from plasmid pSEC:Nuc. An *Nsi*I site was introduced at the 3" end of RBS_{Usp} to allow replacement of the *nuc* gene by a DNA fragment encoding a heterologous protein. pSEC:LEISS:Nuc was constructed by inserting a DNA fragment having *Nsi*I ends encoding LEISSTCDA synthetic propeptide between SP_{Usp} and Nuc into *Nsi*I-linearized pSEC:Nuc. pCYT:L7L12, pSEC:L7L12 and pSEC:LEISS:L7L12 were constructed by replacing the *nuc* gene with the *L7/L12* gene in pCYT:Nuc, pSEC:Nuc and pSEC:LEISS:Nuc, respectively (Fig. 1) (see below for the restriction sites used).

The *B. abortus L7/L12* ribosomal gene was amplified by PCR from *E. coli* expression vector pMal-L7/L12 (28), which encodes an in-frame fusion between L7/L12 and maltose binding protein (MBP). Primers containing one artificial restriction site at each end were constructed according to the *L7/L12* nucleotide sequence (GenBank accession number L19101) (Table 2, oligonucleotides 1 and 2). To secrete L7/L12 protein, we used sense primer (5"-GGATGCATCAGCTGATCTCGCAAAG-3") (the *Nsi*I site is underlined) in which CA (in boldface type) was added just after the *Nsi*I restriction site; the amplified *L7/L12* fragment can be cloned in the same reading frame as SP_{Usp}.

View this table: TABLE 2. Oligonucleotides used in this study
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To produce L7/L12 protein in the cytoplasm or to secrete it in the extracellular medium, the amplified fragment containing *L7/L12* was digested with *Nsi*I and *Eco*RV restriction endonucleases and ligated to *Nsi*I-*Eco*RV-digested pCYT:Nuc and pSEC:Nuc, respectively. In the first construction (pCYT:L7/L12; Fig. 1A), L7/L12 is produced in the cytoplasm. In the latter construction (pSEC:L7/L12; Fig. 1B), L7/L12 protein may be secreted via SP_{Usp}, resulting in the pSEC:L7/L12 plasmid (Fig. 1B).

To obtain fusion protein Nuc:L7/L12, the *Nsi*I-digested fragment containing *nuc* was purified from plasmid pVE3753 and ligated to *Nsi*I-digested pSEC:L7/L12. The resulting plasmid pSEC:Nuc:L7/L12 contains the *nuc:L7/L12* gene fusion (Fig. 1C). For fusion protein LEISS:L7/L12, the pSEC:L7/L12 plasmid was digested with *Nsi*I and *Cla*I enzymes, and the resulting *Nsi*I-*Cla*I fragment containing *L7/L12* was then cloned into *Nsi*I-*Cla*I-digested pSEC:LEISS:Nuc. The resulting plasmid was named

pSEC:LEISS:L7/L12 (Fig. 1D).

To obtain a cell-wall-anchored form of L7/L12, *L7/L12* was cloned into pVE5547 (Stefania Usai, personal communication) using primers 3 and 4 (Table 2). This vector contains the DNA fragment encoding the *Streptococcus pyogenes* M6 protein cell wall anchor region, CWA_{M6}. CWA_{M6} corresponds to the 138 C-terminal amino acids of the mature M6 protein. The PCR fragment containing *L7/L12* was digested with *Sal*I and *Eco*RV enzymes and cloned directly into the *Sal*I-*Eco*RV-digested pVE5547, resulting in p5547:L7/L12 encoding an in-frame fusion of L7/L12 with CWA_{M6} (Fig. 1E).

Purification of L7/L12 protein.
L7/L12 expression was induced by adding 0.6 mM IPTG (isopropyl-ß-D-thiogalactopyranoside) to a culture of an *E. coli* strain containing pMal-L7/L12 (28). Protein extracts were prepared after induction and diluted 1:5 with phosphate-buffered saline (PBS) (pH 8.4). The suspension was then loaded onto an amylose resin column (New England Biolabs) and washed 10 times with PBS, and the fusion protein was eluted with PBS plus 10 mM maltose. The purified fusion protein MBP-L7/L12 was cleaved with the Xa factor that recognizes a specific amino acid sequence between MBP and L7/L12. Although the expected products were observed, Xa digestion appeared to be partial.

Nisin induction.
P_{nisA} induction for L7/L12 expression in the different lactococcal constructs was performed as follows: overnight cultures of *L. lactis* NZ9000 derivative strains were used to inoculate fresh medium at a 1:50 dilution. After 3 h of incubation (corresponding to an optical density at 600 nm of around 0.4), nisin A (Sigma) was added to cultures at a final concentration of 1 ng/ml. Growth was continued for 1 more hour before performing protein extractions.

Protein extraction and Western blot experiments.
For cell fractionation, 2 ml of *L. lactis* cultures was centrifuged for 5 min at 6,000 x *g* at 4°C. Protein extracts were then prepared from exponentially growing cultures according to Le Loir et al. (23).

Western blotting was performed as described (37). After blotting, nonspecific protein binding sites were blocked with a solution containing 1% bovine serum albumin in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.5% Tween 20. The nylon membranes were incubated 2 h with a 1/2,000 dilution of L7/L12 antibodies (Eurogentec). After washing, membranes were incubated for 45 min with protein G-horseradish peroxidase conjugate (Bio-Rad) and signals were detected using an enhanced chemiluminescence (ECL) kit (Dupont-NEN). After ECL detection, different nonsaturated film exposures were scanned by a Scanjet II (Hewlett-Packard) using Deskscan II and ImageQuant programs, and average values were determined. For quantification, signals were compared to those of known amounts of purified L7/L12.

In the case of Nuc:L7/L12 fusions, membranes were dehybridized by washing twice at 65°C, for 30 min, in 200 mM glycine-0.5% Tween 20-0.12 N HCl. Hybridization was then performed with Nuc antibodies kindly provided by J. R. Miller (Lilly Corporate Center, Indianapolis, Ind.).

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Cytoplasmic production of L7/L12 in *L. lactis*.

A cytoplasmic form of L7/L12 was expressed in *L. lactis* NZ9000 using pCYT:L7/L12 (Fig. 1A; Table 1). L7/L12 production was analyzed by Western blot using L7/L12 antibodies (Fig. 2). Proteins from whole cells (noninduced and induced cultures) and supernatants were fractionated. Strain NZ9000(pVE3655) was used as a negative control. No L7/L12 signal was detected in the absence of nisin in strain NZ9000(pCYT:L7/L12) extracts. In induced cultures, a faint band with an apparent molecular mass of ~28 kDa was present in the cell fraction only (Fig. 2). This is about twice the size expected for cytoplasmic L7/L12, and might correspond to either aberrant protein migration or the persistence of a sodium dodecyl sulfate (SDS)-resistant dimer of the cytoplasmic L7/L12 protein. This result shows that cytoplasmic production of L7/L12 with a yield estimated at 0.5 mg/liter can be achieved in *L. lactis*.

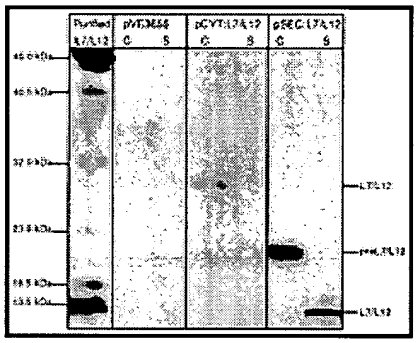

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FIG. 2. Intra- and extracellular production of L7/L12 protein in *L. lactis*. L7/L12 production was estimated by Western blot analysis on exponential-phase cultures of lactococcal strains containing pVE3655 (negative control), pCYT:L7/L12 (encoding the cytoplasmic form) and pSEC:L7/L12 (encoding the secreted form). Protein extractions were prepared on cellular (C) and supernatant (S) fractions of these cultures induced with nisin for 1 h. Purified L7/L12 protein was loaded as standard. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunodetection was performed with anti-L7/12 antibodies. Arrows indicate migration positions of precursor form (preL7/L12) and mature form (L7/12).

SP_{Usp} can drive the secretion of the ribosomal protein L7/L12 in *L. lactis*.

L7/L12 is normally a cytoplasmic protein. Extracellular expression of L7/L12 was obtained by its fusion to the Usp45 signal peptide, SP_{Usp} (Usp45 is the major *L. lactis*- secreted protein) (42). The construct encoding this fusion, pSEC:L7/L12 (Fig. 1B), was introduced into strain NZ9000, and L7/L12 secretion was evaluated by Western blotting (Fig. 2). Two bands were revealed: (i) one major band in the cell fraction corresponds to the preL7/L12 precursor form (preL7/L12) (migration position around 21 kDa for an expected size around 17 kDa); and (ii) a weaker band in the supernatant fraction migrates at the expected size of L7/L12 mature protein (around 14 kDa). This result shows that *L. lactis* can secrete the cytoplasmic protein L7/L12. The total protein yield of the secreted form of L7/L12 (3 mg/liter) was about sixfold greater than that for the cytoplasmic form with a secretion efficiency (SE; percentage of secreted L7/L12 detected in the supernatant) of 35%. The above results show the feasibility of secreting a naturally cytoplasmic protein in *L. lactis*.

L7/L12 SE is low regardless of induction levels.

The precursor accumulation observed above with the secreted L7/L12 form could result from a secretion bottleneck in *L. lactis* due to saturation of the secretion machinery by high-level nisin induction (1 ng/ml) (in this case, processing might be more efficient at lower induction levels) or from incompatibility between intrinsic features of the preL7/L12 precursor and the *L. lactis* secretion machinery (in this case, precursor processing might be hampered regardless of the induction level). To address these hypotheses, growth and L7/L12 production of secreting strain NZ9000(pSEC:L7/L12) were monitored after a 1-h induction with two different concentrations of nisin (0.1 and 1 ng/ml; Fig 3). Growth of the control strain NZ9000(pVE3655) was not affected by nisin addition (data not shown). Also, growth of strain NZ9000(pSEC:L7/L12) cultures with or without nisin was similar, suggesting that L7/L12 precursor production did not slow down growth. Western blots showed that precursor accumulation occurs even at low induction levels (data not shown). This result suggests that the poor SE of preL7/L12 is probably due to intrinsic features of the precursor leading to poor recognition and/or processing by the lactococcal secretion machinery, favoring the second hypothesis.

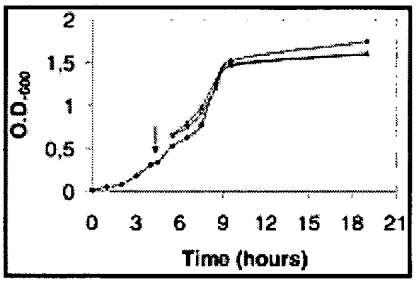

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FIG. 3. Influence of L7/L12 production level on the growth of *L. lactis*. Growth curves of L7/L12-secreting strains NZ9000 (pSEC:L7/L12) after a 1-h induction with different concentrations of nisin (0.1 and 1 ng/ml). The arrow indicates the time of nisin induction (optical density at 600 nm [$O.D._{600}$] = 0.345). Symbols: ♦, without nisin; ■, with nisin at 1 ng/ml; *, with nisin at 0.1 ng/ml.

Production yield of secreted L7/L12 is increased by a fusion with Nuc.

To improve the SE of L7/L12, the *nuc* gene encoding the *Staphylococcus aureus* nuclease (Nuc) was inserted between the DNA fragment encoding the fusion between SP_{Usp} and L7/L12. Nuc is a highly stable, naturally secreted protein that has been successfully used as an export reporter in protein fusions (2, 20, 32-33). Nuc is synthesized as an intracellular precursor that is cleaved in two mature forms, NucB and NucA (23). The plasmid pSEC:Nuc:L7/L12 (Fig. 1C), which encodes the fusion protein Nuc:L7/L12, was transferred into *L. lactis* NZ9000. The resulting strain was streaked on a solid medium containing nisin and tested for Nuc activity (22). All Nuc:L7/L12-secreting *L. lactis* colonies displayed a clear Nuc^+ phenotype (data not shown). Noninduced cells showed weak Nuc activity probably due to background P_{nisA} promoter activity in colonies after overnight incubation (data not shown). Yields of Nuc:L7/L12 were examined by Western blots using either Nuc (data not shown) or L7/L12 antibodies (Fig. 4). In NZ9000- induced cultures, the total amount of fusion protein is estimated to be 2.5-fold higher for pSEC:Nuc:L7/L12 than for pSEC:L7/L12 (8 versus 3 mg/liter, respectively). In the cell fraction, three major bands were detected for Nuc:L7/L12: (i) the highest band corresponds to the preNuc:L7/L12 precursor form; (ii) the second and third bands correspond to mature NucB:L7/L12 and

NucA:L7/L12 forms. Several bands of lower molecular weight present in the cell fraction are likely to correspond to degradation products.

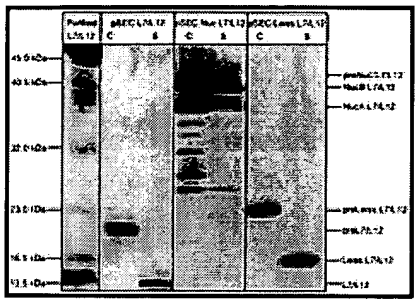

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FIG. 4. Improvement of the secretion of L7/L12 protein in *L. lactis*. Recombinant L7/L12 production was analyzed by Western blot analysis on induced cultures of *L. lactis* NZ9000 strains containing pSEC:L7/12 (encoding the secreted form), pSEC:Nuc:L7/12 (encoding the fusion protein Nuc:L7/L12), or pSEC:LEISS:L7/L12 (encoding the fusion protein Leiss:L7/L12). Protein extractions were prepared on cellular (C) and supernatant (S) fractions of these cultures induced with nisin for 1 h. Purified L7/L12 protein was loaded as standard. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunodetection was performed with anti-L7/12 antibodies. Note that migration of all bands is slower than expected for preNuc:L7/L12 precursor and NucB:L7/L12 and NucA:L7/L12 mature forms (migrating as 46, 44, and 40 kDa for expected sizes around 36, 33, and 30 kDa).

The supernatant fraction contained a major band corresponding to mature NucB:L7/L12. Only a few weak bands corresponding to degradation products were detected in the supernatant, indicating that the secreted fusion is not subject to extracellular proteolytic degradation. Similar patterns were obtained using anti-Nuc antibodies (data not shown). The SEs of Nuc:L7/L12 and L7/L12 were similar (38 and 35%, respectively). These results show that Nuc mature moiety acts as a protein carrier that mainly enhances the production yield of L7/L12.

Synthetic propeptide enhances both SE and production yield.

We previously showed that the synthetic propeptide, LEISSTCDA (hereafter called LEISS), enhanced secretion of heterologous proteins in *L. lactis* (20, 23-24). The effect of LEISS on the SE of L7/L12 protein was examined. LEISS was fused between SP_{Usp} and L7/L12 protein as expressed from pSEC:LEISS:L7/L12 plasmid (Fig. 1D). A comparison of L7/L12 and LEISS:L7/L12 secretion was made by Western blotting (Fig. 4). The SE of LEISS:L7/L12 was significantly improved compared to that observed for L7/L12 (50% for LEISS:L7/L12 compared to 35% for L7/L12 in repeated experiments). Compared to L7/L12 protein alone, production yield increased from 3 to 8 mg/liter. A major effect of LEISS was the increase in the amount of mature LEISS:L7/L12 protein present in the medium compared to L7/L12 protein alone (4 versus 1 mg/liter). Thus, L7/L12 production in *L. lactis* can be optimized by fusion of either a heterologous secreted protein or a synthetic propeptide at the N terminus of the mature protein.

Cell wall anchoring of L7/L12 in *L. lactis*.

A cell-wall-anchored form of L7/L12 was obtained by creating a fusion between L7/L12 and the CWA_{M6} region of *S. pyogenes* M6 protein (expressed from plasmid p5547:L7/L12; Fig 1E). Production of L7/L12:CWA_{M6} in *L. lactis* was analyzed by Western blots in protoplast, cell wall and supernatant

fractions (Fig. 5). About 40% of L7/L12:CWA_{M6} was localized in the cell wall, while 60% remained unprocessed in the protoplast as a band corresponding to preL7/L12:CWA_{M6}. Cell-wall-anchored L7/L12 appeared as several closely spaced bands, a characteristic of cell-wall-anchored proteins; this is attributed to cell surface proteolysis (10). Bands corresponding to smaller proteins may also reflect degradation of anchored L7/L12 by cytoplasmic or surface housekeeping proteases. Two very weak bands were detected in the supernatant, and may correspond to L7/L12:CWA_{M6} released from the cell wall (Fig. 5). The cell-wall-anchored L7/L12 protein obtained here may be a particularly useful candidate for oral vaccine against *B. abortus*.

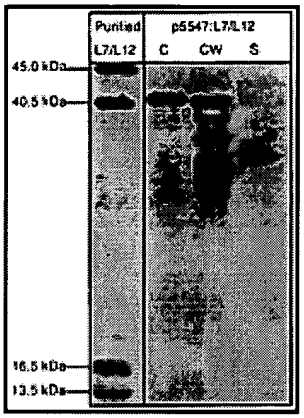

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FIG. 5. Inducible cell wall anchoring of L7/L12 protein in *Lactococcus lactis*. L7/L12 production was estimated by Western blot analysis on exponential-phase cultures of lactococcal strains containing p5547:L7/12 (encoding the fusion protein L7/L12:CWA_{M6}). Protein extractions were performed in cell lysates (C), supernatants (S), and cell wall (CW) fractions of these cultures induced with nisin for 1 h. Purified L7/L12 protein was loaded as standard. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunodetection was performed with anti-L7/12 antibodies. Note that preL7/L12:CWA_{M6} migrates at 38 kDa for an expected size of 30 kDa.

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Efficient targeting and production of the *B. abortus* protective ribosomal antigen L7/L12 (29) were attained for the first time in the food-grade LAB *L. lactis*. The constructed strains will allow us to determine whether recombinant *L. lactis* can be used as L7/L12 delivery vehicles to elicit both mucosal and systemic immune response against brucellosis and which antigen location will induce the most efficient response.

L. lactis has been previously reported to successfully target tetanus toxin fragment C (TTFC) model antigen to cytoplasm, cell wall, and extracellular medium (47, 48). TTFC was shown to elicit an immune and protective response, suggesting that *L. lactis* could be used as a successful delivery vector (26, 35, 48). Furthermore, secretion of interleukin-10 by *L. lactis* was recently shown to have biological activity in mice, although secretion levels are reportedly low (40). These encouraging results suggest that protein export from *L. lactis* may indeed be a feasible mode of antigen delivery.

Since *L. lactis* is not a colonizing commensal bacterium, the chosen approach was to preload the organism in vitro with high levels of heterologous antigen prior to immunization (4, 48). In this work, the *L7/L12* gene was expressed under the control of P_{nisA}, the nisin-inducible lactococcal promoter, to preload the bacteria with antigen before administration. This expression system is now frequently used to achieve high-level heterologous protein production in *L. lactis* (2, 5, 12).

Five *L. lactis* strains were constructed to produce L7/L12 in three different forms: cytoplasmic, anchored and secreted. The cytoplasmic production can protect the antigen from degradation in the upper digestive tract. During intestinal transit, *L. lactis* will then be lysed (11), and the accumulated antigen will thus be released. However, in strain NZ9000(pCYT:L7/L12), L7/L12 was detected in the cellular fraction at only a very low level (0.5 mg/liter). Our hypothesis was that ClpP, the major housekeeping intracellular protease of *L. lactis* (13), degraded the cytoplasmic form of L7/L12. pCYT:L7/L12 was then introduced in a *clpP* mutant of NZ9000 but no significant increase was observed, suggesting that ClpP was not involved in this putative proteolysis (data not shown).

To target a protein at a precise cell location requires specific signals that do not guarantee successful targeting, particularly for cytoplasmic proteins reportedly difficult to secrete (39). When we fused L7/L12 with SP_{Usp}, aiming to direct the secretion of the cytoplasmic protein L7/L12, the production in strain NZ9000(pSEC:L7/L12) was enhanced sixfold compared to intracellular production. After fusion with SP_{Usp}, the same increase was recently observed in the rate of production in *L. lactis* of the nonstructural protein NSP4 of bovine rotavirus (12), the E7 oncoprotein of human papillomavirus (2) and the bovine ß-lactoglobulin protein (5). To explain this enhancement, our hypothesis is that the recognition of the preL7/L12 by the *L. lactis* secretion machinery could allow it to escape intracellular proteolysis even if no degradation band was observed. However, the SE was low (35%), possibly due to inefficient translocation of a cytoplasmic protein, or to inefficient precursor processing; inefficient export of a cytoplasmic protein was previously observed in *E. coli* (8).

To enhance production and secretion of L7/L12 in *L. lactis*, we fused L7/L12 with the staphylococcal nuclease (Nuc). We showed that the fusion with Nuc resulted in a 2.5-fold enhancement of the total amount of fusion protein with a similar SE compared to the secreted L7/L12. Nuc, reportedly strongly immunogenic, could also reveal some carrier activity towards the immunogenicity of L7/L12 and the capacity of L7/L12 to elicit an immune response will be compared when fused or not to Nuc protein.

The synthetic propeptide LEISS described as a secretion enhancer (23, 24) was also used to improve the L7/L12 secretion. LEISS alters the N terminus of the mature fusion protein by introducing two negative charges at positions +2 and +8 (23). Analysis of the peptidic sequence of the N-terminal part of L7/L12 revealed a net global charge of -2 in the first 10 amino acid residues. The observed SE of LEISS:L7/L12 was 50%, compared to 35% for L7/L12. However, the major effect of LEISS was the 2.7-fold increase in the amount of mature LEISS-L7/L12 protein secreted in the extracellular medium in comparison to L7/L12 protein alone.

The fusion of L7/L12 with the cell wall anchor (CWA) region of the *Streptococcus pyogenes* M6 protein

led to an efficient anchoring of L7/L12. This construction will be interesting for in vivo tests since the cell wall anchoring of L7/L12 may be advantageous because the bacterial cell may provide an adjuvant activity that would enhance the immunological response of hosts (46). Another advantage is that the anchored L7/L12 may be less exposed to degrading or denaturing agents such as proteinases or acid-rich environments such as the stomach of man and animals.

Our results provide a means of improving yields and stability of a protein for in vitro and possibly in vivo presentation. They indicate that *L. lactis* is able to produce recombinant L7/L12 protein that possesses antigenic properties. These strains will be very useful to explore new strategies of vaccination against brucellosis. Such a system would represent a good alternative to the commonly studied live bacterial vectors that are mostly derived from pathogenic invasive microorganisms such as *Salmonella* spp., *Vibrio cholerae*, *Mycobacterium* spp., and *B. abortus*.

At present, immunization assays are under investigation with these constructions; this will allow definition of the construction that will give the highest immune response and protection against challenge with *B. abortus*.

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FOOTNOTES

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Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against Brucella abortus infection.

Oliveira SC, Splitter GA.

Department of Animal Health and Biomedical Sciences, University of Wisconsin, Madison 53706, USA.

BALB/c mice were immunized with the recombinant Brucella abortus L7/L1 ribosomal protein fused to maltose binding protein (MBP). Vaccinated animals mounted a specific immune response to the recombinant fusion protein as demonstrated by immunoblot analyses. Additionally, B. abortus L7/L12 ribosomal protein conferred a significant degree of protection when compared to mice vaccinated with adjuvant alone, adjuvant plus MBP or B. abortus. These results indicate that a recombinant B. abortus protein, previously identified as T-cell-reactive, engendered protective immunity to mice against brucellosis.

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Mapping of Chlamydia trachomatis proteins by immobiline-polyacrylamide two-dimensional electrophoresis: spot identification by N-terminal sequencing and immunoblotting.

Bini L, Sanchez-Campillo M, Santucci A, Magi B, Marzocchi B, Comanducci M, Christiansen G, Birkelund S, Cevenini R, Vretou E, Ratti G, Pallini V.

Department of Molecular Biology, Siena University, Italy.

Proteins from purified elementary bodies of Chlamydia trachomatis were separated by two-dimensional gel electrophoresis on nonlinear wide-range immobilized pH gradients in the first dimension and polyacrylamide gradient gels in the second dimension. The maps obtained with this system are highly reproducible and resolve ca. 600 spots. By using immunoblot analysis with specific antibodies and/or N-terminal amino acid sequencing, we established the map positions of a number of described chlamydial proteins, such as the major outer membrane protein (MOMP) the 60 kDa cystein-rich outer membrane protein (OMP2), the DnaK-like, GroEL-like, and macrophage infectivity potentiator (MIP)-like proteins, the plasmid-encoded pgp3 protein two ribosomal proteins (S1 and L7/L12), and the protein-elongation factor EF-Tu. Other proteins, for which gene assignment was not possible, have been identified by three parameters (Mr, pI and N-terminal sequence). This work provides a preliminary basis for a future and progressive compilation o a genome-linked database of chlamydial proteins.

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